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***Toxoplasma gondii*-positive human sera recognize intracellular tachyzoites and bradyzoites with diverse patterns of immunoreactivity**

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Abstract

Antibody detection assays have long been the first line test to confirm infection with the zoonotic parasite *Toxoplasma gondii*. However, challenges exist with serological diagnosis, especially distinguishing between acute, latent and reactivation disease states. The sensitivity and specificity of serological tests might be improved by testing for antibodies against parasite antigens other than those typically found on the parasite surface during the acute stage. To this end, we analyzed the reactivity profile of human sera, identified as positive for anti-*T. gondii* IgG in traditional assays, by indirect immunofluorescence reactivity to acute stage intracellular tachyzoites and in vitro-induced latent stage bradyzoites. The majority of anti-*T. gondii* IgG positive sera recognized both intracellularly replicating tachyzoites and in vitro-induced bradyzoites with varying patterns of immune-reactivity. Furthermore, anti-bradyzoite antibodies were not detected in sera that were IgM-positive/IgG-negative. These results demonstrate that anti-*T. gondii*-positive sera may contain antibodies to a variety of antigens in addition to those traditionally used in serological tests, and suggest the need for further investigations into the utility of anti-bradyzoite-specific antibodies to aid in diagnosis of *T. gondii* infection.

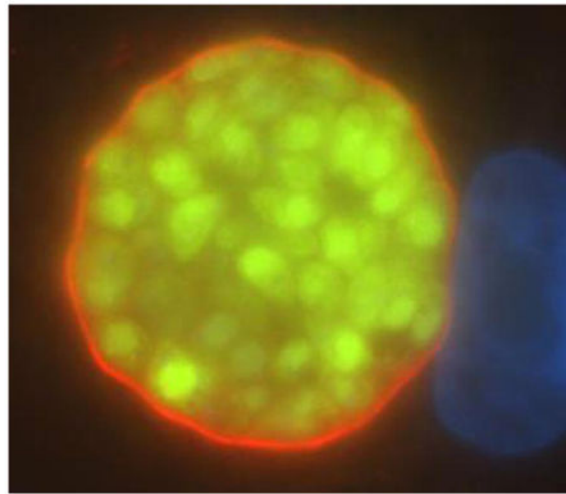
Graphical Abstract

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Keywords

Toxoplasma gondii; Tachyzoite; Bradyzoite; Diagnosis; Serology; Acute infection; Latent infection

1. Introduction

Toxoplasmosis is a parasitic infection caused by the protozoan *Toxoplasma gondii*. Infection may be acquired through ingestion of infective oocysts shed in feline stool or tissue cysts in raw or undercooked meat of chronically infected animals. Infection may also be transmitted vertically by organ transplantation or in utero. During its life cycle, the parasite transitions between stages with differing replicative, metabolic, antigenic and transmissive properties (Dubey, 1998; Bohne et al., 1999; Weiss and Kim, 2000; Gross et al., 2004; Skariah et al., 2010). In the gastrointestinal tract of the definitive host (any feline), the parasite undergoes sexual replication with differentiation into macro- and microgametocytes, and the development of sporozoites in oocysts, which are shed in the feces and are stable in the environment. After oocysts are ingested by an intermediate host (non-feline warm-blooded animal), sporozoites are released and the parasite develops into two forms: tachyzoite and bradyzoite. The tachyzoite is the rapidly replicating, pathogenic stage associated with manifestations of acute disease. Symptoms of acute infection are typically mild in the immune-competent host. However, immune-suppressed individuals are at risk of severe disease and fatal outcomes (Halonen and Weiss, 2013; Jones et al., 2014). Chronic infection occurs due to parasite stage conversion from tachyzoite to bradyzoite, a slowly replicative cell type which survives for long periods in the intermediate host. Bradyzoite conversion is induced by multiple factors including the host immune response, host cell type and metabolism, and the parasite strain (Fux et al., 2007; Ferreira-da-Silva et al., 2008; Weilhammer et al., 2012; Swierzy and Lüder, 2015). While humans can be infected with any of the three major stages (sporozoites, tachyzoites, bradyzoites), serological assays cleared by the United States Food and Drug Administration (FDA) target tachyzoite stage antigens (Boothroyd, 2009) (Supplementary Table S1).

Diagnostic methods for toxoplasmosis include isolation and cultivation of the parasite in an animal model or tissue culture, detection of a serological response to infection, histology and, more recently, molecular detection of parasite-specific nucleic acid (Jorgensen and Pfaller, 2015). Drawbacks to these methods include invasive procedures required to sample potentially infected tissue, the cost of maintaining animals or tissue cultures, and a lack of FDA approved tests for molecular detection. Thus, serological detection of parasite-specific antibodies is typically performed to test for exposure to the parasite, and a negative serological result is thought to exclude the possibility of infection (Montoya, 2002; Press et al., 2005).

A challenge related to serological diagnosis is the difficulty in distinguishing between acute and latent or chronic infection. This is important in guiding the treatment of pregnant women due to the risk of congenital transmission during acute infection. While IgM is typically associated with acute infection, it may not be detected in some acutely-infected individuals and may persist for long periods in others (Fricker-Hidalgo et al., 2013; Beal et al., 2014). Another drawback for IgM testing is the potential for false-positive results (Wilson et al., 1997; Garry et al., 2005). The FDA issued a public health advisory regarding *Toxoplasma* IgM commercial kits in 1997, which included recommendations for follow-up testing at a laboratory with specialized experience in *Toxoplasma* serological testing (Burlington, 1997). Avidity testing is currently recommended to aid in the timing of infection for IgG/IgM-positive pregnant individuals (Jorgensen and Pfaller, 2015). High avidity IgG, typically found in past infections, is useful for ruling out recent infection. However, low avidity IgG, which should be found only in acute or recent infection, has been found to persist long-term in some individuals; this confounds the clinical picture if a single sample is tested (Findal et al., 2015).

One of the major parasite antigens recognized by the human immune system is the surface protein SAG1/p30 (Kasper et al., 1983; Santoro et al., 1985). Accordingly, commercially available serology assays test for antibodies (IgG, IgM) to major surface antigens of the tachyzoite (Supplementary Table S1). Since *T. gondii* is an obligate intracellular pathogen, many antigens are produced during replication within the host cell and are exposed to the immune system upon host cell lysis. Whether antibodies against other antigens play a role in the humoral response against *Toxoplasma*, especially putative ones from intracellularly replicating tachyzoites and latent bradyzoites, is under-explored. To investigate this possibility, we tested human sera positive for anti-*T. gondii* antibodies by an immune-fluorescence assay for immune-reactivity to intracellularly replicating tachyzoites and in vitro switched bradyzoites. Our results demonstrate that anti-*T. gondii*-positive sera may contain antibodies to a variety of antigens in addition to those traditionally used in serological tests. The detection of antibodies to parasite structures found in host cells infected with tachyzoites and bradyzoites opens new avenues to investigation of the humoral response to *Toxoplasma* infection.

2. Materials and methods

2.1. Serum samples

Samples used in this study were remnants of human sera that had been tested at the Indiana University (IU) Health Pathology laboratory (IUHPL), USA for IgG to *T. gondii* by enzyme-linked fluorescent assay (ELFA) ($n = 89$ study samples of 818 clinical samples tested in 2014) (Vidas, bioMérieux, Durham, NC, USA) and for IgM by an IFA ($n = 18$ study samples of 341 clinical samples tested) (Hemagen Diagnostics, Columbia, MD, USA) by routine laboratory protocols and stored at -20°C . Two study specimens were positive for both IgG and IgM. Sample selection criteria were IgG and/or IgM positivity, availability of stored specimen and sufficient residual volume (> 0.25 ml). Clinical laboratory test results were recorded with patient age and gender, and samples were de-identified for further investigations. This study was granted exempt status upon protocol review by the Indiana University School of Medicine Institutional Review Board (IRB).

2.2. Host cell and parasite maintenance and reagents

Human foreskin fibroblasts (HFF, purchased from American Type Culture Collection (ATCC), Manassas, VA, USA) were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 units of penicillin/100 μg of streptomycin per mL, in a humidified incubator at 37°C and 5% CO_2 . Green monkey kidney cells (Vero) were grown under the same conditions. Parasite strains were maintained by passage through HFFs in normal culture medium and grown in Vero cells for all studies using human sera. All experiments were performed with *T. gondii* strain PRU C32 (Singh et al., 2002). This strain carries GFP under the control of the bradyzoite stage-specific *ldh2* promoter.

2.3. Immunofluorescence assays and western blots

For IFA, 1×10^4 PRU C32 tachyzoites were inoculated into Vero cells on coverslips for IFA. After 35 h of incubation, coverslips were fixed with 4% paraformaldehyde, and an IFA was performed using a routine laboratory protocol (Arrizabalaga et al., 2004). The primary antibody was the human serum (1:500 for IgG sero-positive specimens, 1:20 for IgM sero-positive specimens) and the secondary antibody was Alexa-fluor 594 conjugated goat anti-human IgG or anti-human IgM (1:2000 for both classes) (ThermoFisher Scientific, Waltham, MA, USA). DAPI (1:1000) was used to stain nuclear material. In vitro bradyzoites were induced with alkaline stress (RPMI pH 8.2) incubation for 4–5 days in an ambient-air incubator (Weiss et al., 1995) and IFA performed as for tachyzoites. Slides were examined in a zig-zag pattern on a Nikon microscope at $600\times$ total magnification. For each serum sample tested, photomicrographs were taken from three fields of view with parasitic structures observable by phase contrast microscopy. Sera were classified as positive for anti-*Toxoplasma* antibody if specific signal was observed for parasite-associated structures as identified by phase contrast. Staining patterns were compared with previously published patterns for different parasite antigens (cyst wall (Tobin et al., 2010), matrix (Zhang et al., 1999), bradyzoite surface (Saeij et al., 2008), parasitophorous vacuole (PV) membrane (PVM) (Karsten et al., 1998), surface antigen 1 (SAG1), PV-internal structures/intravacuolar network (IVN) (Rome et al., 2008). For in vitro bradyzoites, only GFP-positive structures were imaged. The 4 μm scale bar was determined from the narrowest (non-dividing)

tachyzoite or bradyzoite cell width (reported as approximately 2 μm (Dubey et al., 1998)) in each image and doubling the measurement.

Co-localization experiments were performed with human serum and mouse anti-SAG1 (Genway Inc., San Diego, CA, USA), mouse anti-GRA7 (dense granule antigen 7, (Coppens et al., 2006)) for intracellular tachyzoites, and mouse anti-BAG1 (bradyzoite antigen 1, Weiss et al., 1992; McAllister et al., 1996) antibodies and *Dolichos biflorus* agglutinin (lectin that recognizes cyst wall, Vectorlabs Inc., Burlingame, CA, USA).

For western blots, extracellular parasites were filtered, washed in PBS, and pelleted by centrifugation. The parasite cell pellet was lysed in SDS sample buffer with a final concentration of 5×10^7 tachyzoites/ml. Vero cells were trypsinized, centrifuged, washed with PBS, and lysed in SDS sample buffer at a final concentration of 5×10^6 cells/ml. Ten μl of each lysate were used for western blot per routine laboratory protocol (Garrison and Arrizabalaga, 2009); primary antibody was IgG sero-positive human serum or anti-SAG1-positive or -negative mouse serum (1:5000) and the secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-human IgG or Goat anti-mouse IgG (1:20,000) (Thermo Fisher Scientific). Chemiluminescent substrate (SuperSignal West Femto Substrate, Thermo Fisher Scientific) was applied to the membranes and signal detected by a myECL Imager (Thermo Fisher Scientific).

2.4. *Toxoplasma gondii* serology assays commercially available in the U.S

The U.S. FDA in vitro diagnostic device database, Device@FDA, website (<http://www.accessdata.fda.gov/scripts/cdrh/devicesatfda/index.cfm>) was queried for “*Toxoplasma*” and the resulting table listing FDA documents was exported into Microsoft Excel. Documents (510(k)) for assays listed since the year 2000 were examined for test antigen and test limitations (Supplementary Table S1).

3. Results

3.1. Test population

In 2014, a total of 818 anti-*Toxoplasma* IgG tests were performed at IUHPL, with an average positivity of 16.4%. The IgG sero-positivity rate was 13.6% (48/354) in females and 18.2% in males (83/457) ($P < 0.01$ by student's t -test). Seven samples did not have gender specified, and one of those tested IgG-positive. The highest test volume occurred in patients in the sixth decade of life, and sero-positivity increased with age; patients >70 years of age had the highest rate of sero-positivity (26%) (Supplementary Fig. S1A,B). Eighty-nine anti-*Toxoplasma*-positive serum samples were selected for analysis based on specimen availability and sufficient residual volume (0.25 ml). The study samples included 38 (43%) from females aged 12 to 71 years, and 51 (57%) from males aged 15 to 75.

3.2. Human serum recognizes a diversity of antigens in intracellular tachyzoites

To determine whether IgG sero-positive samples could detect distinct antigens on intracellular tachyzoites, we performed immunofluorescence assays with 89 unique ELFA-positive sera against intracellular parasites grown in Vero cells. As a positive control we used

mouse antibodies raised against the *Toxoplasma* surface antigen SAG1, which has been previously described as a dominant antigen in toxoplasmosis (Kasper et al., 1983; Santoro et al., 1985). Four ELFA IgG-negative human sera were screened by IFA for use as negative controls. One of these specimens demonstrated SAG1-like immune-reactivity while the other three were negative and displayed non-specific signal with prolonged exposure times; one of these was subsequently used as the negative control for future experiments. Because this study was not designed to compare the sensitivity of IFA against intracellular tachyzoites with current diagnostics, no further negative sera were tested. All but one of the serum test samples (88/89) demonstrated immuno-reactivity against intracellular tachyzoites at the dilution tested (1:500). Interestingly, we found a variety of staining patterns when using the IgG sero-positive sera (Fig. 1B). The predominant immuno-staining pattern was strong staining of the parasite membrane, which is reminiscent of what is seen with SAG1 antibodies (Fig. 1B - Mouse α -SAG1). Of the 88 samples that stained intracellular parasites, 84 showed the SAG1-like pattern (Fig. 1B - Human SAG1-like, Table 1). Of those, 24 showed additional staining including the PVM (Fig. 1B - Human SAG1/PV-like), and structures within the PV. Surprisingly, four of the 88 positive samples did not stain the tachyzoite membrane and, instead, showed signal consistent with that of proteins localized within the PV (Fig. 1B - Human PV-internal). The parasite surface and PV staining patterns were confirmed by co-staining with antibodies against SAG1 and the PV localized protein GRA7 (Supplementary Fig. S2).

To further characterize the IgG sero-positive sera, two representative samples of the three IFA staining pattern types shown in Fig. 1 were also tested by western blot (Fig. 2). All sera that exhibited SAG1-like staining by IFA recognized a protein migrating at ~30 kDa (Fig. 2 black arrowhead), similar to that observed with a mouse anti-SAG1 antibody. Nonetheless, in all cases other proteins were detected by the sera, and the 30 kDa protein was not the most prominent. This suggests that antibodies against other surface antigens may play a role in the human response to toxoplasmosis. On the other hand, sera with a PV-internal-like pattern and no IFA reactivity against the parasite surface did not have a detectable band at 30 kDa (SAG1) at the tested dilution (Fig. 2D). Interestingly, both of the two independent serum samples that showed a PV-internal-like pattern by IFA recognized a common set of three proteins (Fig. 2 white arrowheads).

3.3. Sera from *Toxoplasma*-infected humans recognize bradyzoite cysts

While it is thought that the majority of antibodies generated during toxoplasmosis are formed against the tachyzoite stage, we wondered whether sera from IgG sero-positive humans would recognize antigens expressed by the encysted bradyzoite stage. Accordingly, we induced bradyzoite development in tissue culture and tested the ability of all 89 ELFA-positive samples to detect encysted parasites by IFA. Interestingly, the majority of sera (86/89) demonstrated immuno-reactivity to in vitro bradyzoites by IFA (Table 1, Fig. 3B). The major staining pattern was the putative cyst wall, indicating that, although considered latent, the bradyzoite cyst can be detected by the immune response. Other staining patterns observed were similar to bradyzoite surface staining and cyst matrix (Fig. 3B - Bradyzoite surface, Cyst-internal). While the possibilities of remnant SAG1 expression in tissue cultures of bradyzoite cysts or of cross-reactivity with SAG1-like antigens cannot be excluded, a

mouse SAG1-specific antibody demonstrated no signal against tissue culture bradyzoites as expected (data not shown) and anti-*Toxoplasma* negative sera demonstrated no reactivity to in vitro bradyzoites (Fig. 3B - Negative). The different patterns observed with the human sera were confirmed by dual immunofluorescence studies using either antibodies against the bradyzoite-specific protein BAG1, which stain the individual parasites within the cyst, or *Dolichos biflorus* agglutinin, which binds to the cyst wall (Supplementary Fig. S3).

3.4. IgM detection of tachyzoites and bradyzoites

Because a selection process occurs during antibody affinity maturation (Eisen, 2014), we reasoned there might be different antigens detected by IgM during acute infection compared with IgG during chronic infection. To this end, we tested IgM-positive sera for immunoreactivity to intracellular tachyzoites and in vitro bradyzoites. Total anti-*Toxoplasma* IgM testing was performed on 341 specimens in 2014 at IUHPL, with 25 of those testing positive (7.3%). Remnant serum was available from 18 unique IgM-positive patient samples. These were originally determined to be IgM-positive by IFA against extracellular tachyzoites with titers from 1:10 to 1:80. Two of these samples also tested positive for anti-*Toxoplasma* IgG by ELFA. It is unknown whether any samples had avidity testing performed. IgM-positive specimens were from 13 females aged 9 to 59 years, and five males aged 6 to 70 years. We used these samples to perform IFA against intracellularly replicating tachyzoites and in vitro bradyzoites, and used secondary antibodies specific to human IgG or IgM. IgM-specific reactivity towards intracellular tachyzoites was observed in 8/18 specimens, 8/18 had equivocal reactivity, and 2/18 specimens were negative (Table 2). The immunofluorescence patterns by IgM staining were similar to the PVM alone and combinations of the PVM/PV-internal/SAG1 (Table 1). While some IgM-positive sera were also IgG-positive by IFA, no IgM-specific immuno-reactivity was detected against in vitro bradyzoites (Table 2, Fig. 4). One of two IgM-positive specimens that also tested positive for IgG by hospital ELFA also showed IgG-specific reactivity against in vitro bradyzoites, with a pattern similar to cyst wall (Fig. 4). None of the samples which were IgM-positive/IgG-negative by clinical diagnostic testing had detectable immunofluorescence against in vitro bradyzoites, consistent with an initial antibody response directed against tachyzoites.

4. Discussion

The *T. gondii* serology tests commercially available in the U.S. have gone through the 510(k) clearance route by the FDA, which is used for demonstrating substantial equivalence between assays. This suggests that despite advances in technology, including automated testing platforms and chemiluminescence or fluorescence detection methods, serological tests for *T. gondii* have not changed substantially during the past 30 years. This may be due to the acceptable performance of current tests and the challenges in performing clinical diagnostic trials for acute and reactivated toxoplasmosis. Nonetheless, the first commercially available test for *T. gondii* IgG avidity admits test performance has not been established for prenatal screening, testing in newborns, immunocompromised patients, or in cases of reinfection. Further studies with clinical evaluations of performance in these at-risk patient populations are needed.

To improve upon and expand beyond current assays, a more comprehensive understanding of antigens for the different stages of the parasite is needed. By testing anti-*Toxoplasma gondii* IgG-positive sera against intracellularly replicating tachyzoites, we observed a variety of immuno-staining patterns, which suggests that infected individuals produce antibodies to diverse antigens, in addition to SAG1. While the majority of sera tested had immuno-reactivity similar to SAG1, i.e., surface staining of parasites, a small subset of samples demonstrated a staining pattern similar to the PV space. Consistent with this idea, these sera also had different band patterns recognized by western blots. Antigens from the intravacuolar network and PVM are likely exposed to the immune system upon host cell lysis; additionally, dense granule proteins are secreted from both intracellular and extracellular parasites (Nam, 2009). Previous studies have demonstrated a protective effect for antibodies to dense granule proteins in infected mice (Desolme et al., 2000; Cha et al., 2001; Jongert et al., 2007). However, it is not known whether the presence of antibodies to these antigens has a protective effect in human infection.

One explanation for the diversity of antigenic reactivity could be cross-reactivity with antibodies against related parasites. The closest phylogenetic relative to *T. gondii*, *Neospora caninum*, has not been associated with human infections, however infections occurring in domestic or wild animals may lead to human exposure and a serological response (Dubey et al., 2007). Few human serological surveys for *N. caninum*-specific antibodies have been conducted and sero-positivity rates from 0% to 38% have been reported in various populations (Nam et al., 1998; Petersen et al., 1999; Tranas et al., 1999; Lobato et al., 2006; McCann et al., 2008). While we cannot rule out the possibility that observed immunoreactivity is due to cross-reacting antibodies specific for *N. caninum*, previous reports have indicated a lack of cross-reactivity between major surface antigens of *T. gondii* and *N. caninum* (Howe and Sibley, 1999; Zhang et al., 2011).

Recent investigations into antigens suitable for *Toxoplasma* diagnostic methods have utilized multiple approaches including testing for antibodies to secreted antigens and various recombinant parasite antigens (Saadatnia et al., 2011; Holec-Gasior, 2013; Khanaliha et al., 2014). These studies have also demonstrated variable activity between acute and chronic infections and, overall, improved sensitivity when multiple antigens are included in the assay. As the parasite replicates, it manipulates host cell gene expression to promote nutrient acquisition and disrupt the innate immune response (Laliberté and Carruthers, 2008; Blader et al., 2015). Thus, it is also possible that the antibodies are detecting host proteins, i.e. are auto-antibodies. However, we failed to observe specific reactivity to uninfected host cells at the dilution tested by western blot, which suggests the bands recognized are unique to infected cells and are either parasite-specific or a host protein that is upregulated in infected cells.

Tissue cysts are predominantly found in immune-privileged sites such as the brain and skeletal muscle. Here we observed the common presence of anti-bradyzoite antibodies in human sera positive for anti-*T. gondii* IgG. While it is possible immune-reactivity is due to shared or related epitopes between stages, the striking differences between IFA patterns observed against tachyzoites and in vitro bradyzoites indicates a sufficient level of differentiation and recognition of stage-specific antigens. These findings, suggestive of a

humoral immune response to tissue cysts, are in contrast to an earlier study which suggested a minimal humoral response to the tissue cyst stage (Zhang et al., 1995). More recent studies, however, have also detected bradyzoite-specific antibodies from positive sera of humans and chronically infected mice (el-Shennawy et al., 2000; Di Cristina et al., 2004). DNA vaccine studies have shown a protective effect for surface antigen genes specific to the bradyzoite stage, which suggests a bradyzoite-specific response is conducive to controlling the chronic stage of the infection in mice (Zhang et al., 2013). Elucidating the contribution of the humoral immune response in controlling acute and chronic toxoplasmosis will require further work in animal models.

Finally, the observation that anti-*Toxoplasma* IgM-positive/IgG-negative sera lack antibodies to in vitro bradyzoites, in combination with the observation that nearly all of the ELFA anti-*Toxoplasma* IgG-positive sera contain antibodies to in vitro bradyzoites, is intriguing. However, the small sample size and possible false-positive IgM results limit broad generalizations. Inaccuracies with IgM testing have been previously described and inclusion of additional IgM antigens may not increase specificity (Wilson et al., 1997; Garry et al., 2005). The timing of anti-bradyzoite antibody development in humans and the role of humoral immunity in controlling latent infection remain open questions for future investigation. A study of the timing of antibody development in mice infected orally with either oocysts or tissue cysts indicated early responses to sporozoite and bradyzoite antigens (Dö kaya et al., 2014). However, it is possible that cyst wall-specific antibodies would not develop until later in oral infection as ingested cysts are dissolved by gastrointestinal secretions and the parasites rapidly convert into tachyzoites.

Since this study was primarily conducted to investigate the presence of anti-*Toxoplasma* antibodies to additional antigens, study limitations include the absence of additional relevant clinical and laboratory findings associated with acute or reactivated toxoplasmosis. These include lymphadenitis, imaging studies, pregnancy status, anti-*Toxoplasma* IgG avidity, HIV status, CD4 count, etc. Furthermore, while it is generally assumed that a negative result is due to the absence of specific antibodies, another possibility for a negative serology result is hypogammaglobunemia. These results offer clinical evidence to support further investigations into the utility of incorporating additional parasite antigens in order to improve detection of parasite-specific antibodies and to determine if the detection of anti-bradyzoite antibodies can serve as a useful tool to distinguish between acute and chronic toxoplasmosis. Additional studies, including identification of target antigens and testing the recognition of recombinant proteins with well-characterized sera, are needed to demonstrate the clinical utility of expanding the targets included in diagnostic assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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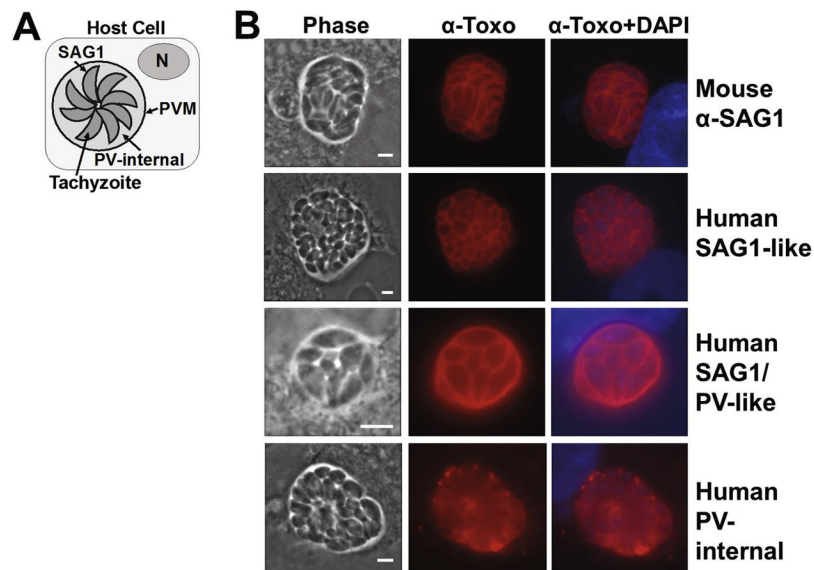
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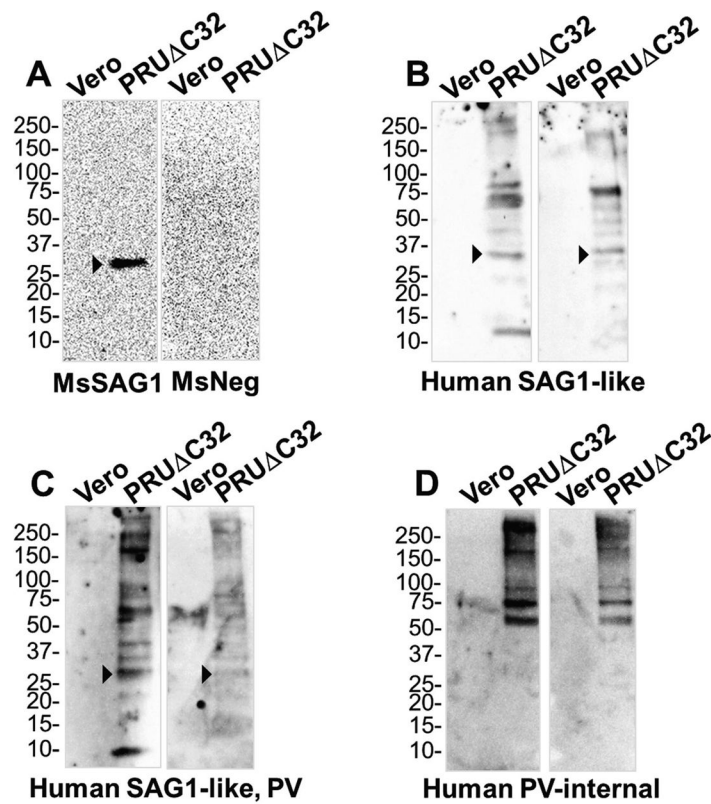
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Highlights

- We screened anti-*Toxoplasma gondii* IgG+ or IgM+ human sera for detection of additional antigens.
- Anti-*T. gondii* IgG+ sera showed immune reactivity to intracellular tachyzoites.
- The IgG+ sera were also immune-reactive to in vitro induced bradyzoites.
- Immune reactivity to in vitro bradyzoites was not observed in IgM+/IgG– sera.
- Future investigations of these antigens are needed to demonstrate clinical utility.

**Fig. 1.**

Human sera positive for anti-*Toxoplasma* antibodies demonstrate a variety of immunofluorescence patterns against intracellular tachyzoites by indirect immunofluorescence (red fluorescence, goat anti-human IgG AF594; blue fluorescence, DAPI). (A) Diagram of tachyzoite-infected cell with arrows indicating immune-reactive structures: N, host cell nucleus; PV, parasitophorous vacuole; PVM, PV membrane; SAG1, surface antigen glycoprotein1. (B) Representative images of each staining pattern are shown. Some sera had combinations of the above patterns (Table 1). Estimated scale bar = 4 μ m.

**Fig. 2.**

Human sera demonstrating different IFA patterns were observed to have different patterns by western blot. Two sera with representative IFA patterns were tested by western blot against uninfected cells and cells infected with *Toxoplasma* tachyzoites. (A) Mouse anti-SAG1 (MsSAG1) and uninfected mouse sera (MsNeg). (B) Human sera with SAG1-like IFA. (C) Human sera with SAG1-like and parasitophorous vacuole (PV) IFA. (D) Human sera with PV-internal IFA. Black arrowheads indicate potential SAG1 (p30) bands; white arrowheads indicate bands common to PV-internal-like staining. Molecular mass indicated in kDa.

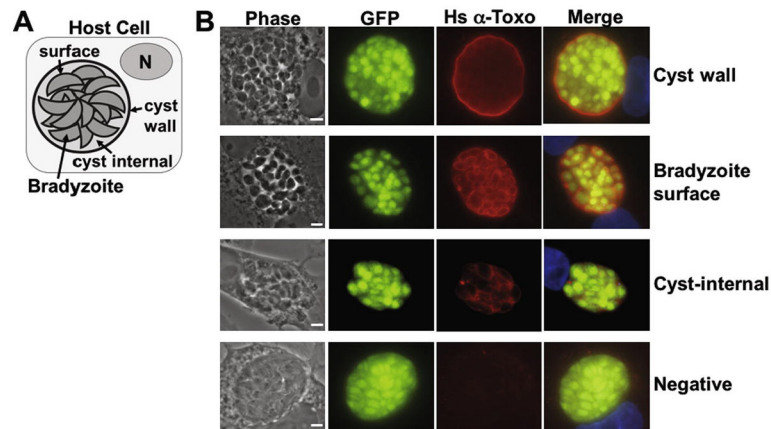


Fig. 3.

Indirect immunofluorescence with human sera (Hs) positive for anti-*Toxoplasma* antibodies demonstrates a variety of immuno-fluorescence patterns against in vitro bradyzoites. In vitro bradyzoites detected with GFP fluorescence (under the control of a bradyzoite-specific promoter (*Idh2*); red fluorescence, goat anti-human IgG AF594; blue fluorescence, DAPI). (A) Diagram of bradyzoite-infected cell with arrows indicating immuno-reactive structures; N, host cell nucleus. (B) Representative images of three staining patterns from three positive sera and one negative sera are shown. Cyst wall, structure enclosing bradyzoites; bradyzoite-surface, bradyzoite cell surface; cyst-internal, structures inside the cyst wall and outside the bradyzoites. Some sera had combinations of the above staining patterns (Table 1). Estimated scale bar = 4 μ m.

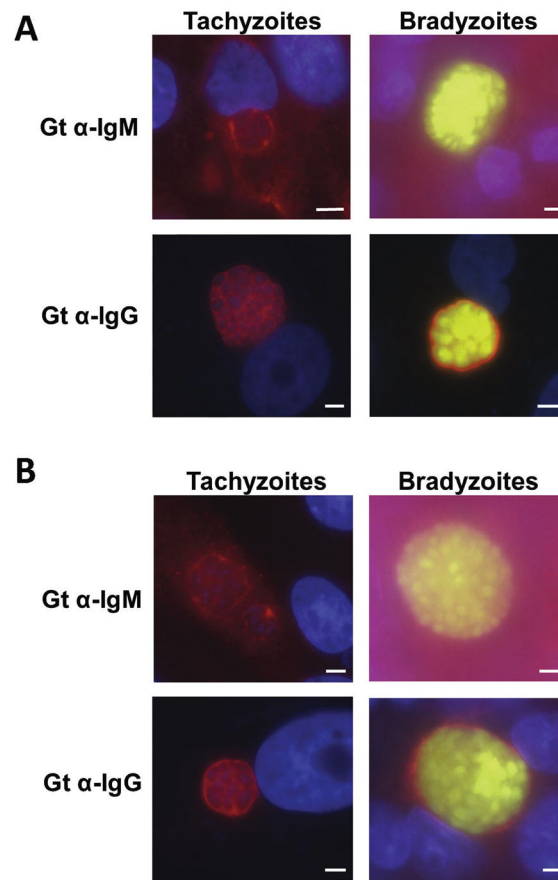


Fig. 4.

Representative images of IFA with two human sera (A, B) that demonstrated IgM- and IgG-positive immunoreactivity against intracellular tachyzoites as well as IgG-positive reactivity to in vitro bradyzoites. Primary antibody, human serum; secondary anti-sera (goat, Gt) specific for human IgM or IgG (red fluorescence, goat anti-human IgG AF594 or goat anti-human IgM AF594; green fluorescence, GFP; blue fluorescence, DAPI). Estimated scale bar = 4 μ m.

Table 1

Combinations of immunofluorescence patterns directed towards tachyzoites and in vitro bradyzoites by human serum positive for anti- *Toxoplasma gondii* IgG antibodies.

Tachyzoite	Bradyzoite	N (%)
Tz surface	Bz surface/cyst wall/cyst internal	41 (46.1)
Tz surface/PVM/PV - internal	Bz surface/cyst wall/cyst internal	14 (15.7)
Tz surface	Bz surface	12 (13.5)
Tz surface	cyst wall	7 (7.9)
Tz surface/PVM/PV - internal	cyst wall	6 (6.7)
PV-internal	cyst wall	3 (3.4)
Tz surface/PVM/PV - internal	Bz surface	2 (2.2)
Tz surface	negative/equivocal	2 (2.2)
PV-internal	Bz surface	1 (1.1)
negative/equivocal	negative/equivocal	1 (1.1)
Total		89 (100)

Tz, tachyzoite; Bz, bradyzoite; PV, parasitophorous vacuole; PVM, PV membrane.

Table 2

IFA results for 18 IgM-positive serum samples tested for IgG or IgM-specific reactivity to intracellular *Toxoplasma* tachyzoites and in vitro bradyzoites.

IFA Antigen	Secondary antibody					
	Goat anti IgG			Goat anti IgM		
	Pos. ^a	Equiv. ^b	Neg. ^c	Pos. ^a	Equiv. ^b	Neg. ^c
Primary antibody sera						
Intracellular tachyzoites						
ELFA IgG+/IFA IgM+	2	0	0	1	0	1
ELFA IgG-/IFA IgM+	5	3	8	7	8	1
In vitro bradyzoites						
ELFA IgG+/IFA IgM+	1	0	1	0	0	0
ELFA IgG-/IFA IgM+	0	0	16	0	0	16

^aPositive (Pos.) samples demonstrated parasite-specific immunoreactivity in 3/3 randomly imaged microscopic fields.

^bEquivocal (Equiv.) samples had immunoreactivity in 1–2 of three fields.

^cNegative (Neg.) samples displayed no specific immunoreactivity in any imaged field.